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p53 and NF-κB Co-regulate Pro-inflammatory Gene Responses in Human Macrophages

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Abstract

Macrophages are sentinel immune cells that survey the tissue microenvironment, releasing cytokines in response to both exogenous insults and endogenous events such as tumorigenesis. Macrophages mediate tumor surveillance and therapy-induced tumor regression; however, Tumor Associated Macrophages (TAMs) and their products may also promote tumor progression. Whereas NF-κB is prominent in macrophage-initiated inflammatory responses, little is known about the role of p53 in macrophage responses to environmental challenge including chemotherapy or in TAMs. Here, we report that NF-κB and p53, which generally have opposing effects in cancer cells, co-regulate induction of pro-inflammatory genes in primary human monocytes and macrophages. Using Nutlin-3 as a tool, we demonstrate that p53 and NF-κB rapidly and highly induce IL-6 by binding to its promoter. Transcriptome analysis revealed global p53/NF-κB co-regulation of immune response genes including several chemokines, which effectively induced human neutrophil migration. Additionally, we show that p53, activated by tumor cell paracrine factors, induces high basal levels of macrophage IL-6 in a TAM model system (Tumor-conditioned Macrophages [TCMs]). Compared to normal macrophages, TCMs exhibited higher p53 levels, enhanced p53 binding to the IL-6 promoter and reduced IL-6 levels upon p53 inhibition. Taken together, we describe a mechanism by which human macrophages integrate signals through p53 and NF-κB to drive pro-inflammatory cytokine induction. Our results implicate a novel role for macrophage p53 in conditioning the tumor microenvironment and suggest a potential mechanism by which p53-activating chemotherapeutics, acting upon p53-sufficient macrophages and precursor monocytes, may indirectly impact tumors lacking functional p53.

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Keywords

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Introduction

The immune system is intricately connected to various stages of tumorigenesis. On one hand, it is critical in the clearance of senescent and damaged tumor cells after therapy and in tumor prevention through detection and elimination of early stage cancer cells ([1, 2]). Work from Xue *et al* ([1]) is exemplary of this phenomenon, since they show that p53 stabilization triggers senescence in tumor cells in mice and subsequent activation of innate immune cells that ultimately clear the senescent tumor cells, leading to tumor regression. On the other hand, immune cells associated with the tumor microenvironment of advanced cancers have tumor-promoting functions through secretion of soluble factors that promote survival, proliferation, angiogenesis, and metastasis ([3]). Underlying central components to this dual function of the immune system in tumorigenesis are macrophages.

Macrophages are key players in innate immunity, and their functions depend on the environment in which they reside. In a non-cancerous microenvironment, macrophages are central detectors of infectious and non-infectious exogenous stress including DNA damaging agents such as chemotherapeutics. Upon stimulation, macrophages trigger cascades of cell-cell signaling that result in synthesis and secretion of pro-inflammatory cytokines and chemokines, and, consequently, recruitment of other effector immune cells. In the context of a tumor, tumor cells secrete soluble factors that recruit and program Tumor Associated Macrophages (TAMs) to support tumor growth ([3]). TAMs are phenotypically distinct from classical macrophages in that they exhibit different morphology and expression markers. For example, TAMs have high expression of Interleukin-6 (IL-6), CXCL1, Interleukin-8 (IL-8) and CCL2 ([3–5]). The secretion of cytokines and chemokines constitute a major mechanistic feature of macrophage function; therefore, understanding the precise mechanisms that drive the induction of pro-inflammatory genes is crucial.

Nuclear factor-κB (NF-κB) plays an essential role in inflammation, innate immunity, and cancer ([6, 7]). Activated by inflammatory stimuli such as pathogen-associated molecular patterns (PAMPs) and various cytokines including tumor necrosis factor-alpha (TNF-α), NF-κB enhances transcription of several pro-inflammatory cytokines such as IL-6 and IL-8, which are secreted from the cell and propagate the immune response by acting on neighboring immune cells ([6]). Additionally, NF-κB is found constitutively activated in several types of human cancers and has been shown to promote cancer cell growth and survival, for example, by regulating the transcription of anti-apoptotic genes ([7]).

Another master regulator of stress response, the tumor suppressor p53, also has roles in inflammation and immunity ([8, 9]). Recently, we reported that p53 can upregulate most members of the Toll-like receptor (TLR) family and consequently enhance TLR-dependent production of pro-inflammatory cytokines ([10, 11]). Surprisingly, p53 regulation of the TLRs is restricted to human cells since the p53 response elements (p53REs) in the TLR promoter regions are not conserved in mice ([10]), suggesting that some p53-related immune responses

can only be addressed in human material. These results highlight the fact that p53 has an important physiological role in the immune system in addition to its well-characterized role as a tumor suppressor, providing a new dimension to the broad role that p53 plays in human biology.

Mechanistically, activation of p53 and NF-κB is similar and involves stress-induced degradation of inhibitors. Specifically, Mdm2 (murine double-minute 2 or human hMdm2) binds to p53 and targets p53 for proteasomal degradation. During cellular stress, Mdm2 and p53 are modified and can no longer bind to each other, leading to p53 stabilization ([12]). For NF-κB, the subunits are held in the cytoplasm through binding to IκB (Inhibitor of nuclear factor-κB) proteins. Upon stimulation, IκB is phosphorylated by the upsteam IKK (inhibitor of nuclear factor-κB kinase) complex, leading to ubiquitination and degradation of IκB, which allows the NF-κB subunits to enter the nucleus and modulate transcription (reviewed in ([6])). Although both transcription factors are activated by similar stimuli, the resulting biological consequences are generally considered to be opposing in that NF-κB signaling is mostly associated with pro-survival cues and p53 induction is pro-apoptotic ([13]). Additionally, signaling cross-talk between p53 and NF-κB is antagonistic ([13]). However, there are a few examples of p53 and NF-κB working together ([14, 15]).

Since macrophages are key players in the immune response to tumor cells, we have addressed the potential role for p53 regulation of immune genes and possible interactions with the NF-κB network within human macrophages from healthy human subjects and in a TAM *in vitro* model system, Tumor Conditioned Macrophages (TCMs). We show that p53 and NF-κB co-regulate the expression of several pro-inflammatory genes including cytokines (such as IL-6) and chemokines (such as CXCL1) in TCMs and in normal macrophages in response to p53 and NF-κB activating agents. Taken together, these results identify a unique p53/NF-κB interaction within macrophages that we propose may be important in amplifying the immune responses associated with various stages of tumorigenesis and after chemotherapy and radiotherapy.

Materials and Methods

Primary macrophages

Alveolar macrophages were isolated as previously described ([16]). Monocyte-derived macrophages ([17]) and neutrophils were harvested with Histopaque-1077 (Sigma-Aldrich) from the blood of healthy volunteers as previously described ([17]). For TCMs, monocytes were cultured with 50% conditioned media from MDA-MB-231 cells for the first 4 days as previously described ([5]). (IRB #: 10-E-0063)

Reagents

The following reagents were used: 10 μM Nutlin-3, 0.3 μM Doxorubicin, 300 μM 5-Fluorouracil, 50 μM Pifithrin-α, 50 μM Bay-11-7082 (Sigma-Aldrich); 100 ng/mL LPS (InvivoGen); p65 S276 blocking peptide or control peptide (Imgenex, per manufacturer's protocol).

Chromatin-Immunoprecipitation assay

ChIP assays were performed as described in our previous work ([18]). SYBR green assays were performed using IL-6 specific primers (Cat. # NM_000600.2 (-)01Kb: GPH1011803(-)01A; Qiagen Inc.).

Real time PCR

Taqman gene expression assays or SYBR green assays were run with Applied Biosystems 7900HT Fast Real-Time PCR System and results were analyzed with SDS2.4 software. Primers are described in the supplement.

Microarray Analysis

Microarrays were performed with the Affymetrix GeneChIP Instrument system using procedures previously described ([18]) and analyzed using a mixed linear ANOVA model:, where Y_{ijkl} represents the log base 2 intensity value for the *l*th observation on the *i*th treatment for the $_j$th subject and $_k$th biological replicate. μ is the grand mean expression and ε_{ijkl} represents the random error. The errors are assumed to be normally and independently distributed with mean 0 and standard deviation δ for all measurements. Thresholds used for detecting differentially expressed genes: FDR < X, FC +/− Y. Treatment is a fixed effect whereas subject and replicate are random effects. The following contrasts were performed to compare mean intensity values: LPS vs. Non-Treated; Nutlin vs. DMSO. GEO accession: GSE43596.

ELISAs

Secreted IL-6 and CXCL1 protein from treated macrophages was quantified using the IL-6 Human ELISA Kit from Life Technologies and the Human CXCL1/GROa Immunoassay from R&D Systems following the manufacturers' protocol.

Immunoblotting assays

Macrophages were lysed in RIPA buffer for whole cell extracts or with NE-PER kit (Thermo Scientific) for nuclear and cytoplasmic extracts, and immunoblots were performed as described in ([10]). Specific antibodies are listed in the supplement.

Neutrophil Migration Assay

The QCM Chemotaxis Cell Migration Assay (EMD Millipore) was used following the manufacturer's protocol. Neutrophils were seeded at 20×10^4 cells per well and allowed to migrate for 2 hours. Fluorescence was measured as relative fluorescence units (RFU).

Statistical Analysis

For analysis of expression changes (treated compared to untreated or vehicle control), ChIP binding, and neutrophil migration, the data were log transformed and then a one sample t-test was performed for each pair to test if the mean fold change is ≤ to 0, or alternatively, the mean is > 0. Assuming a normal distribution with mean = μ and variance σ^2, test statistic is defined as:

$$t_c = \frac{\overline{Y} - \mu_0}{\hat{\sigma}^2} = \frac{\sqrt{n}(\overline{Y} - \mu_0)}{S}$$

where is the mean, *n* is the sample size, and *S* is the standard deviation of the values. We set equal to 0 and perform the test at $\alpha = 0.05$. Significance (indicated with an *) is determined if t_c > the critical value of a *t*-distribution with n-1 degrees of freedom and α=0.05.

Results

Activation of p53 induces IL-6, TNFα and IL-8 expression

In our initial experiments, we assessed the effect of p53 activation on gene expression in primary human alveolar macrophages using the chemotherapeutic drugs DOX and 5FU and the small molecule Nutlin-3, which stabilizes p53 ([19])(Fig. S1A). Treatment with DOX highly induced mRNA levels of the cytokine gene IL-6, a pro-inflammatory gene important to macrophage function (Fig. 1A). As shown in Figure 1B, Nutlin-3 treatment for 6 hours also enhanced mRNA levels of the cytokines IL-6 and TNFα and the chemokine IL-8 in macrophages from every donor tested; however, there were large differences among the genes and donors. The expression of the *p21* gene, a well-known p53 target that identifies p53 activation, was also increased by Nutlin-3 treatment (Fig. 1B) but with less variation. Notably, IL-6 mRNA was induced in some donors to much higher levels than TNFα or IL-8 (Fig. 1B). Since IL-6, TNFα, and IL-8 protein were all induced (Fig. 1C & S1B), p53 activation is likely to have a strong functional impact.

Rapid induction of IL-6 by Nutlin-3

Due to low cell recovery and availability, experiments with human alveolar macrophages are limiting. Therefore, we utilized macrophages differentiated *ex vivo* from primary human blood monocytes (monocyte-derived macrophages). Similar to results with alveolar macrophages, induction of IL-6 after treatment with DOX, 5FU, and Nutlin-3 was also observed in monocyte-derived macrophages (Fig. 2A). All three treatments also induced p21, demonstrating p53 responsiveness (Fig. 2A). Since IL-6 induction was the highest and most consistent cytokine product among donors after Nutlin-3 treatment, our subsequent experiments focused on Nutlin-3 activation of p53.

Since p53 nuclear accumulation is observed within 1 hour of Nutlin-3 treatment (Fig. S1A), we assayed the kinetics of IL-6 induction by Nutlin-3 in monocyte-derived macrophages. IL-6 mRNA induction levels were measured after 2, 4, and 6 hour Nutlin-3 treatments of macrophages from each donor (Fig. 2B). Within 2 hours the level of IL-6 mRNA was dramatically increased, up to 10,000-fold, in cells of all but one donor (which showed an ~8 fold induction). The rapid and large increases are similar to cytokine production triggered through TLR signaling by PAMPs such as LPS ([20]). Additionally, IL-6 induction by Nutlin-3 treatment was dependent on transcription since pretreatment with Actinomycin D, a transcription inhibitor, abolished IL-6 mRNA induction (Fig. S1C).

The kinetics and level of IL-6 induction by Nutlin-3 varied considerably among donors, suggesting large differences in individual responses to stresses that activate p53. For example, macrophages from donors “117” and “120” were high responders with IL-6 induction levels in the 10,000–25,000 fold range, whereas “123” and “125” were relatively lower responders with induction levels reaching 10–100 fold (Fig. 2A–B). There was much less variability in the kinetics of p21 induction compared to IL-6 induction by Nutlin-3 (Fig. 2A–B), suggesting that the differences are not due to p53 level.

The very rapid and dramatic IL-6 induction by Nutlin-3 was surprising since this deviates from the typical kinetics of p53-dependent gene regulation described in the literature. Whereas gene regulation by other transcription factors such as NF-κB can be rapid (within minutes) ([21]), p53 regulation of transcription generally occurs more slowly (over several hours) ([22]). In addition, the increases in protein levels for IL-6, IL-8 and TNFα after Nutlin-3 treatment were comparable to the levels induced by LPS treatment, a well-known stimulator of the inflammatory response and activator of NF-κB (Fig. S1B). To confirm that the induction of IL-6 by Nutlin-3 was p53-dependent, we examined the effects of the p53 inhibitor pifithrin-α (PFT-α) ([23]). (Several attempts to significantly reduce p53 using siRNA approaches were unsuccessful due to low transfection efficiency of primary human macrophages. Additionally, as reported by Liu *et al.* ([24]) and from our preliminary experiments, Nutlin-3 treatment alone did not induce IL-6 expression in bone marrow derived macrophages from wild type mice, preventing our use of p53−/− murine macrophages in this study.) As expected, PFT-α reduced Nutlin-3-induced p21 levels in all donors (Fig. S1D). Since, as shown in Figure 2C, the PFT-α pretreatment dramatically lowered Nutlin-3-induced IL-6 mRNA levels, we conclude that p53 is required for IL-6 induction by Nutlin-3 in human macrophages.

Induction of IL-6 by p53 activating agents is limited to the monocyte/macrophage cell lineage

Several other types of primary cells and cancer cell lines were tested for the effect of p53 activating agents on IL-6 induction. As shown in Table S1, short (*e.g.*, 2 hours) and long (*e.g.*, 24 hours) incubations with Nutlin-3, DOX, and 5FU did not induce IL-6 expression in cancer cell lines of various origins and p53 status, such as breast (MCF-7, $p53^+$), bone (Saos2, $p53^-$ and U2OS, $p53^+$), lung (H1299, $p53^-$) and colon (HCT-116, $p53^+$). As expected, p21 was up-regulated in all cell lines carrying a functional *TP53* gene. Other types of human primary cells were also treated with Nutlin-3, DOX, and 5FU, including human diploid fibroblasts, $CD3^+$ T-lymphocytes, etc. (Table S1). Whereas all of these cells showed p53 responsiveness (*i.e., p21* induction ([10])), the only human primary cells that showed induction of either IL-6, TNFα or IL-8 after treatment with p53 activating agents were $CD14^+$ monocytes, which are macrophage precursors. Altogether, p53 regulation of IL-6 appears restricted to human monocytes and macrophages.

Activation of NF-κB is required for Nutlin-3-induction of IL-6 in primary macrophages

Having established that p53 is required for Nutlin-3 induction of IL-6, we next investigated whether NF-κB is also required. First, NF-κB is a well-known regulator of the transcription of the pro-inflammatory genes IL-6, TNFα, and IL-8 ([25, 26]). Second, the kinetics of IL-6

induction by Nutlin-3 are comparable to those for cytokine induction by PAMPs, which are largely driven by NF-κB ([20]). Third, there are reports describing cooperation between p53 and NF-κB in the transcription of certain genes ([15, 27]). Finally, NF-κB and p53 are known to be coordinately activated by certain stimuli, such as DNA damage, oxidative stress and immune stimuli ([28]).

We first evaluated whether NF-κB is indeed activated by Nutlin-3 in human macrophages. In the canonical NF-κB pathway, the inhibitor IκBα is phosphorylated and degraded, which frees the NF-κB subunits to translocate to the nucleus to promote transcription of target genes including the IκBα gene ([6]). We found that IκBα protein decreases within 30 minutes of Nutlin-3 addition to monocyte-derived macrophages, followed by an increase to almost basal levels at 6 hours (Fig. 3A). Similar results were seen with alveolar macrophages (Fig. S2A). Additionally, IκBα phosphorylation on Ser32 is seen as early as 30 minutes after Nutlin-3 treatment ("pIκBα," Fig. 3A).

In addition to regulation by the inhibitor IκB proteins, NF-κB subunits are further regulated by post-translational modifications such as phosphorylation. Serine 276, an important activating phosphorylation site of the NF-κB subunit p65, ([29]) is phosphorylated after a 2 hour treatment of Nutlin-3 (Figure 3A, "pp65"). Cytokine induction by nutlin requires phosphorylation of both IκBα and p65, as IL-6 induction is markedly reduced by pretreatment either with Bay-11-7082 (Fig. 3B), a small molecule that inhibits IκBα phosphorylation and p65 nuclear translocation ([30]), or with a p65 decoy peptide that specifically competes with and blocks phosphorylation of S276 on the endogenous p65 protein (Fig. S2B). Thus, Nutlin-3 represents a useful tool to probe the effect of dual p53 and NF-κB activation on gene regulation in macrophages.

Nutlin-3 treatment induces p53 and p65 binding to the promoter region of IL-6

In silico analysis of the IL-6 gene promoter region revealed a potential p53 response element (p53RE) consisting of a decamer half-site located 362 base pairs (-362bp) upstream from the TSS in addition to a previously reported κB site (-138 bp) ([31]) (Fig. 3C). Since we previously established p53 responsiveness at half-sites ([32]), we performed ChIP assays to determine if p53 and NF-κB could cooperate *in cis* to mediate IL-6 expression. As depicted in Figure 3D–E, both p53 and p65 bound to the promoter of the IL-6 gene after one hour of Nutlin-3 treatment of monocyte-derived macrophages. However, when compared to p65, occupancy of p53 was lower; the levels of DNA pulled down by the p65 ChIP were 0.7–1.25% of the input as compared to 0.3–0.5% of the input for p53 ChIP. One possible explanation for weaker p53 binding is that the p53RE is a half-site, which generally shows less binding than full-sites ([32]). As expected, p53 and p65 also bind to the p21 promoter (Fig. S2C), as previously shown in other cell systems ([33, 34]). Taken together, these data show that both p65 and p53 bind to regions near the IL-6 gene, suggesting that they work together *in cis* to regulate IL-6 gene transcription and expression.

Chemokines are highly induced by both Nutlin-3 and LPS

We hypothesized that if p53 and NF-κB co-regulate IL-6 gene transcription in human macrophages, there is likely a larger set of genes that are also subject to the co-regulation.

To investigate this possibility, we performed microarray analyses with monocyte-derived macrophages from two different donors treated with either LPS or Nutlin-3 for 2 hours. We reasoned that since LPS is a well-known activator of NF-κB, genes regulated by both LPS and Nutlin-3 might have responses similar to that of IL-6. We chose to use macrophages from donors with similar demographics (Fig. S3A).

Principal component analysis showed that the microarrays were of good quality and that even with similar demographics the donors grouped separately for all treatments (Fig. S3B). Nonetheless, gene regulation in response to 2 hour treatments with either Nutlin-3 or LPS was remarkably similar in both donors as shown in the heat map in Figure S3C. Although most studies have addressed p53 networks with Nutlin-3 at later times ([35]), we also find elements of p53 signature (such as induction of p21 and Mdm2) at the much shorter time of 2 hours in macrophages. Since our goal was to identify genes regulated like IL-6, we chose to focus only on the up-regulated genes with a threshold 1.7-fold change (Nutlin-3/DMSO or LPS/Untreated). Strikingly, most of the genes up-regulated by Nutlin-3 (155/175) were also up-regulated by LPS (Fig. 4A). Thus, by using Nutlin-3 as an agent to activate p53 and LPS to identify NF-κB-driven changes in gene expression, we identify a subset of 155 genes that are candidates for cooperative regulation by p53 and NF-κB.

Gene ontology analysis of the gene subset up-regulated by both Nutlin-3 and LPS revealed that ~40% of these up-regulated genes could be classified as functioning in the immune response where immune and inflammatory response functions were the most significant (with the lowest p-values) as determined by the g:profiler program ([36]) (Table S2). Importantly, if genes within this subset are indeed regulated in a similar manner as IL-6, then there should be response elements for NF-κB and p53 near these genes. In a search for conserved transcription factor binding sites 1 kB upstream of the TSS for each gene, we found several that would be capable of binding NF-κB subunits (Table S3). The motif with the most significant p-value was the p65 motif (p-value of $1.58e^{-7}$; Table S3). Since conventional prediction programs are poor identifiers of p53REs, we used the “p53 Scan” software to search for predicted p53REs and found that a large subset of the 155 genes had associated p53REs (40 out of the 155 genes), and 25 out of the 155 genes had both associated p53RE and κB sites (Table S4).

Further classification of the 155 gene subset based on the level of fold-change revealed that several of the genes were highly upregulated (greater than 10-fold) similar to IL-6 (Fig. 4B). As expected, IL-6, IL-8, and TNFα were highly induced by both Nutlin-3 and LPS (Table S5). Additionally, chemokines including CXCL1, CCL20, IL-8, CCL4, and CXCL3 were among the highest induced genes after Nutlin-3 treatment (Table S5, the * refers to chemokines). Induction of these chemokines was validated with RT-PCR (Fig. 4C–D), and several of these chemokines have potential p53REs, some of which are listed in Table S6. Some genes, like CXCL3, have multiple p53REs. Additionally, CCL5 and CCL3 had perfect (*i.e.*, no mismatches) half-site p53REs (bold letters; Table S5). p53 and NF-κB are both required for Nutlin-3 induction of these chemokines, since inhibition of p53 or NF-κB by pretreatment with PFT-α or Bay-11-7082, respectively, significantly reduced Nutlin-3 induced CXCL3, CCL3, and CCL4 levels (Fig. S3D). Together, our data suggest that these chemokines are regulated similarly to IL-6 in primary human macrophages.

Nutlin-3 treatment of macrophages enhances chemokine secretion and induces neutrophil migration

Having established that activation of p65 and p53 in primary human macrophages enhances the expression of specific cytokines and chemokines, we investigated the biological consequences. Since chemokines such as CXCL1, IL-8 and CXCL3 secreted from macrophages can bind to receptors on the surface of neutrophils to promote migration ([37]), we tested whether Nutlin-3 treatment increased chemokines in the culture and enhanced neutrophil migration. As shown in Fig. 5A and Fig. S1B, treatment of macrophages with Nutlin-3 and LPS (used as a positive control) for 24 hours induced robust CXCL1 and IL-8 secretion.

To determine if these secreted chemokines are functional, a migration assay was performed in order to test whether the chemokine-rich culture supernatant resulting from Nutlin-3 treatment was capable of promoting neutrophil migration. Migration of primary human neutrophils toward supernatants from macrophages treated for 24 hours with Nutlin-3 or LPS was significantly higher than the untreated and DMSO control supernatants (Fig. 5B). Taken together, these data show co-regulation of cytokine and chemokine expression by p53 and NF-κB in primary human macrophages. While the role of NF-κB in this process is well-described, our data highlights a novel role of p53 in macrophage function.

p53 is activated and drives IL-6 expression in Tumor Conditioned Macrophages

Having identified a subset of chemokines and cytokines regulated by p53 in normal macrophages, we next assessed whether p53 plays a similar role in gene regulation in TAMs. To address this issue, we used an established *in vitro* model system of TAMs in which human peripheral blood monocytes (PBMCs) or isolated monocytes are differentiated into macrophages by culturing them with conditioned media from cultured cancer cells lines or primary tumor cells resulting in Tumor Conditioned Macrophages (TCMs) ([4, 5]). Confirming successful induction of TCMs, macrophages cultured under these conditions exhibited a distinct, elongated morphology compared to normal macrophages cultured under standard conditions ("MAC") and showed upregulation of several TAM/TCM markers such as IL-6, IL-8, CXCL-1, and CCL2 (Fig. 6A–B) ([38]). Interestingly, most of these TAM/TCM markers including IL-6 are genes we had identified as co-regulated by p53 and NF-κB and have also been shown to contribute to tumorigenesis ([4, 5, 39]). However, while NF-κB activity has been shown to regulate the expression of these genes in TAMs/TCMs ([3]), the role of p53 in this process is unknown. We first tested whether p53 was activated in TCMs and whether it contributes to their high basal expression levels of IL-6. As shown in Fig. 6C, p53 protein levels are indeed higher in TCMs compared to normal MACs. Furthermore, four classical p53 targets, p21, Mdm2, Puma, and Bax, were consistently increased between 2- and 10-fold in TCMs compared to MACs depending on the gene and donor, suggesting that the stabilized p53 is functional. Interestingly, whereas p53 activation generally occurs through stabilization of p53 protein, the TCMs showed increased p53 mRNA compared to MACs, which could contribute to the larger level of p53 proteins (Fig. S4A). Furthermore, p53 binding to the IL-6 promoter region was increased in TCMs, and treatment with PFT-α for four hours reduced IL-6 levels in TCMs (Fig. 6E–F). Interestingly, additional p53 stabilization by treatment with Nutlin-3 did not induce IL-6 in TCMs, which might be due to

high IL-6 basal levels (Fig. S4B). Taken together, these data indicate that p53, activated by paracrine factors released by tumor cells, promotes IL-6 expression in TCMs.

Discussion

We have identified a novel role for p53 that is specific to the regulation of several pro-inflammatory genes in human macrophages, including IL-6, IL-8 and CXCL1. Importantly, NF-κB co-activation is essential for this regulation. While previous investigations have suggested the potential for p53/NF-κB interactions([15]), our study is the first to investigate such interactions in human primary immune cells. Importantly, the only cells in which we were able to detect p53/NF-κB co-regulation of cytokines were macrophages and precursor monocytes. Presented in Fig. 7 is a model summarizing our finding on potential p53 and NF-κB responses in normal macrophages and TAMs. In macrophages associated with normal tissue, enhanced cytokine/chemokine induction due to activated p53 and NF-κB may lead to neutrophil recruitment and inflammation. Given that p53 and NF-κB are activated by many exogenous stimuli, this co-regulatory transcriptional mechanism might serve as an important amplifying element in the macrophage's response to a wide array of tissue-damaging insults. In the tumor microenvironment, macrophage p53 appears to be activated in response to paracrine tumor signals and, in part, facilitates IL-6 expression. In this context, the tumor-derived growth factors may induce a p53 response in the macrophages, which in turn alters cellular behavior of the tumor cells. This p53 response could possibly have some beneficial consequences in tumors, which are often p53 defective. On one hand, p53 may function to promote senescence, especially since p53 and NF-κB have been shown to cooperate in senescence and NF-κB is important in cytokine/chemokine gene regulation in TAMs ([3, 40]). On the other hand, the pro-tumor effects of IL-6 and chemokines including CXCL1 on tumor cell survival, angiogenesis and recruitment of pro-tumor Tumor Associated Neutrophils (TANs) have been well-described ([3]). Thus, p53 within TAMs might contribute to tumor promotion through supporting a paracrine axis of secreted cytokines and chemokines (including IL-6 and CXCL1) between TAMs and tumor cells. The impact of activated p53 on cytokine/chemokines in TAMs along with the tumorigenic consequences as described in our model will be of interest in future studies.

Given the rapid kinetics of pro-inflammatory gene induction and the high levels of IL-6 induction, our findings may also be more broadly relevant to tissue responses induced during p53-activating cancer therapies. For example, circulating IL-6 is detected early after radiotherapy and can be used as a predictive diagnostic test for radiation pneumonitis ([41, 42]). Here, we found that the level of IL-6 induction by p53 and NF-κB activation was among the highest of all cytokines and occurred within 2 hours in macrophages from all donors. Our finding that the magnitude of IL-6 induction is donor-dependent implies that there may be underlying genetic determinants of IL-6 activation by p53 and NF-κB. For example, there may be polymorphisms in one or both of these transcription factors or their response elements. Our finding that the induction of IL-6 was dramatically more variable than that of the classical p53 target gene p21 may suggest that polymorphic p53 response elements in the IL-6 promoter are likely. We propose that such genetic variants, if identified, could perhaps be used as predictors of the inflammatory response to chemotherapy or radiation therapy.

This is the first report to our knowledge showing that Nutlin-3 activates NF-κB. While the mechanism of Nutlin-3 activation needs to be further defined, it may be through the DDR. Dey *et al.* ([43]) have reported that Nutlin-3 can inhibit rather than enhance NF-κB function via a p53-dependent mechanism; however, that was found in lung cancer cells. Nutlin-3 activation of NF-κB is likely cell type-dependent and may depend on whether Nutlin-3 initiates the DDR, which also appears to be determined by cell type and Nutlin-3 dose ([44–47]). Furthermore, Nutlin-3 and a similar small molecule, RITA, which has also been shown to induce the DDR ([48]), are currently being evaluated for cancer therapy and may promote macrophage-mediated inflammatory responses that could serve as adjuvants in anti-cancer responses.

An intriguing finding in our study is that p53 and NF-κB work together to regulate gene expression, contrary to most previous studies. There have been a few reports of small degrees of p53 and NF-κB cooperativity ([14, 15]) unlike that seen here, and the nature of the p53/NF-κB is likely context-dependent ([27]). Most of the research describing the opposing interaction between p53 and NF-κB has been conducted in either human cancer cells or mouse primary cells. Few reports have addressed p53/NF-κB interactions in primary human cells, especially in relation to macrophage function during chemotherapy. Our finding that pro-inflammatory gene induction by Nutlin-3 is limited to primary human macrophages and monocytes highlights the fact that interactions between p53 and NF-κB are different depending on context, *e.g.*, cancer cells vs. noncancerous “normal” cells and lymphocytes vs. macrophages. The dramatic and specific responsiveness of macrophages identifies programmatic differences from other cells, possibly to assure ROS-enhanced immune responses. Since tissue macrophages are also relatively resistant to genotoxic injury ([49]), our results suggest that tissue macrophages may serve a dual function as sensors and as effectors of an amplified response to genotoxic injury. Expression of p53 in response to DNA damage could potentiate NF-κB-induced inflammatory response and immune cell recruitment. The underlying mechanisms for assuring these programmatic differences between macrophages and other cell types as well as the absence in rodents are intriguing issues that have larger regulatory as well as evolutionary implications.

Another unexpected finding is that p53 is stabilized in TAM-like macrophages and enhances IL-6 expression. While the roles of wild type and mutant p53 are well-described in tumorigenesis, there have been no studies to our knowledge addressing p53 functions in tumor-associated cells like TAMs. p53 is commonly mutated in tumor cells; however, TAMs presumably have wild type p53 since they originate from circulating monocytes that are recruited into the tumor microenvironment by soluble factors ([3]). This suggests that the role of p53 in TAMs is likely different from its role within tumor cells. Here we show that p53 is activated in the absence of exogenous stress in TAM-like cells, but the mechanism of this activation is currently unknown. Since NF-κB is activated in TAMs and can bind to κB sites in the *TP53* gene to regulate p53 transcription ([3, 50]), one possibility is that NF-κB enhances p53 transcription in TAMs. It is also possible that signaling within the macrophage by tumor cell-secreted soluble factors like M-CSF and CCL2 ([4]) activates p53 independently of NF-κB. Furthermore, the role of p53-driven pro-inflammatory gene expression in TAMs is unclear. As mentioned above, p53 within TAMs could conceivably have pro-tumor

functions. However, it is also possible that macrophage p53 could promote senescence in TAMs, as secretion of cytokines and chemokines is associated with senescence (Senescence Associated Secretory Phenotype [SASP]) and there is continuous turnover of TAMs in the tumor microenvironment ([3]). Thus, the mechanism of p53 activation in TAMs, the functional role of p53 in TAMs, as well the role of TAM p53 in tumorigenesis warrant further investigation.

In summary, our results show that p53 and NF-κB together drive expression of cytokines and chemokines including IL-6 and CXCL1 in macrophages. The secretion of these factors may have distinct biological effects depending on the microenvironment, promoting inflammation in normal tissues upon injury and modifying cancer cell responses in the tumor microenvironment. Importantly, we found that Nutlin-3 had no apparent effect on the expression of IL-6 in TAM-like cells, possibly because p53 is already present. This may suggest that systemic treatment with p53-activating chemotherapeutics might enhance anti-tumor responses of normal macrophages without augmenting pro-tumor functions of TAMs. The net effect of TAM p53 upon tumorigenesis remains to be defined. Nonetheless, our studies suggest that therapies based on p53-activation may impact tumor cell biology through p53-positive macrophages even in patients with tumors lacking functional p53.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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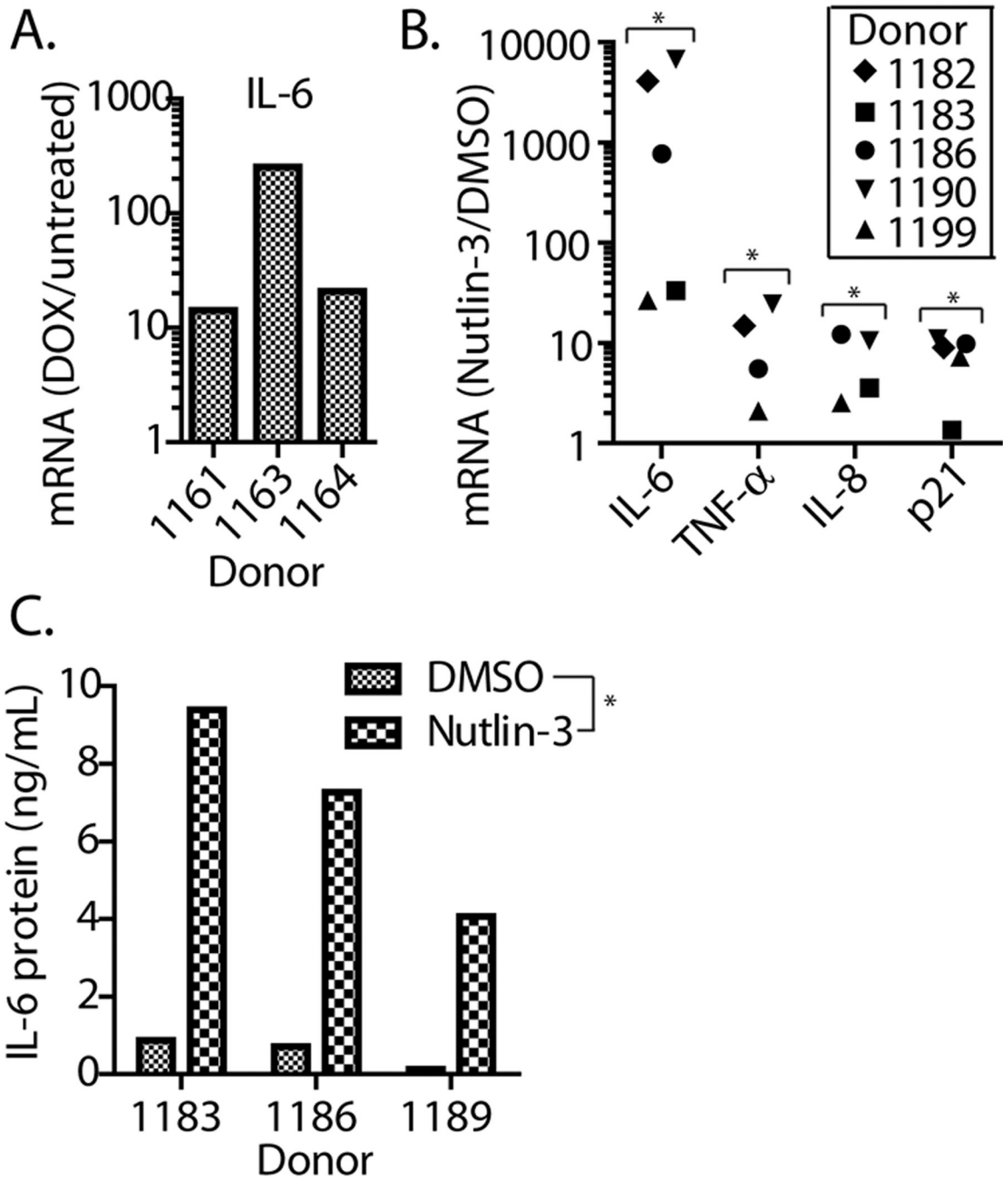

Figure 1. DOX and Nutlin-3 treatment induces expression of pro-inflammatory genes including IL-6

A) Alveolar macrophages from different donors were treated with DOX (0.3 μM) for 24 hours, and mRNA levels of IL-6 were measured with RT-PCR; values were significantly increased compared to untreated controls ($p<0.05$). B) mRNA levels of IL-6, TNFα, IL-8, and p21 of alveolar macrophages treated with Nutlin-3 (10 μM) for 6 hours were measured with RT-PCR, and values were normalized to DMSO treated controls. mRNA levels of each gene for the group of donors were significantly (indicated with an *) increased with Nutlin-3 treatment compared to DMSO ($p<0.05$). C) Secreted IL-6 protein from alveolar macrophages treated with either DMSO or Nutlin-3 for 6 hours was measured with an ELISA. Values from each donor are

plotted, and, when grouped, IL-6 mRNA levels from Nutlin-3 treated samples are statistically increased compared to DMSO ($p<0.05$). Each donor is identified with an arbitrary number indicated in the graphs.

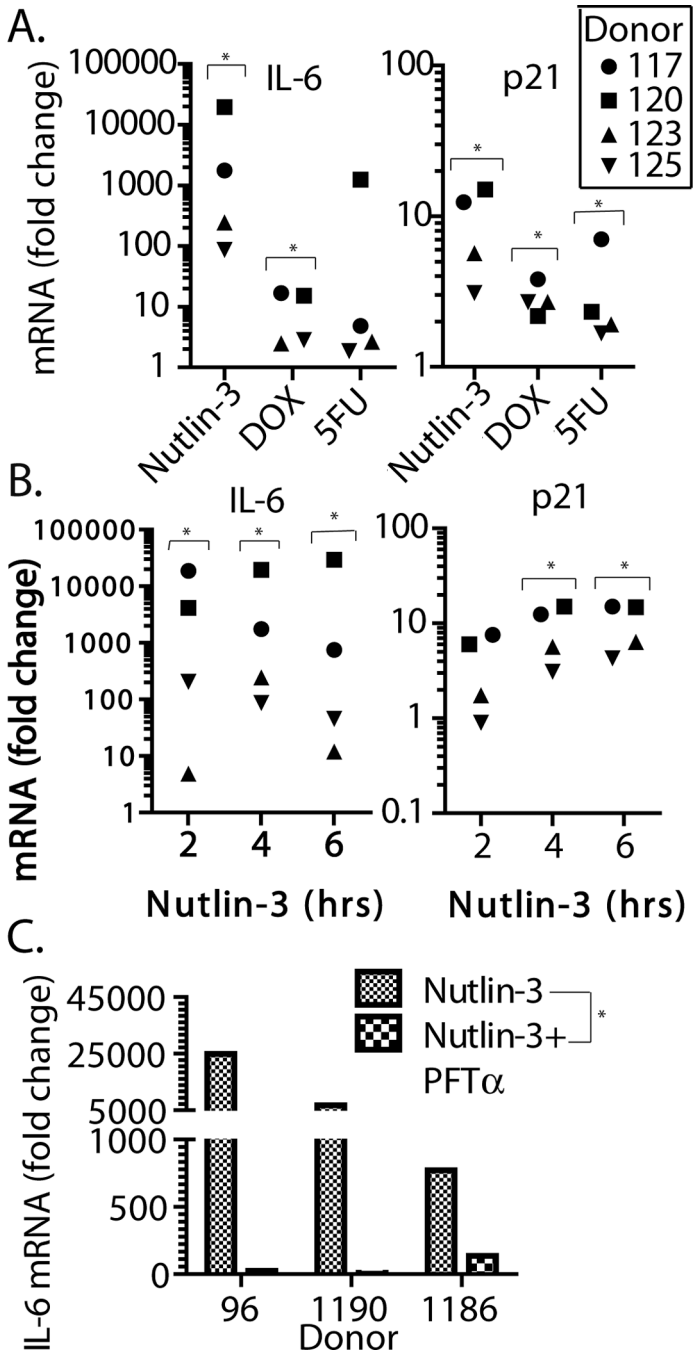

Figure 2. p53 activation induces rapid and high IL-6 expression in monocyte-derived macrophages

A) Monocyte-derived macrophages were treated with Nutlin-3 or DOX for 4 hours or with 5FU for 6 hours, and IL-6 and p21 mRNA levels were measured with RT-PCR. B) IL-6 and p21 mRNA levels of monocyte-derived macrophages were measured with RT-PCR after treatment with Nutlin-3 for the indicated times. For A) & B), values were normalized to DMSO treated controls for Nutlin-3 and 5FU or untreated controls for DOX. Each donor in A) and B) is identified with an arbitrary number indicated in the A) graph. C) Fold-change in IL-6 mRNA levels was measured from monocyte-derived macrophages (Donor 96) and alveolar macrophages (Donors 1190 and 1186) treated with Nutlin-3 for 6 hours with and without Pifthrin-α pretreatment (4 hours,"PFT-α"). Values were normalized to DMSO or PFT-α (alone) treated controls. $p<0.05$ for expression changes in all

panels except for the following: p=0.058 for IL-6 induction by 5FU (2A); and p=0.063 for p21 induction after a 2 hour Nutlin-3 treatment (2B, 2 hrs). Statistical significance (*) is indicated for the group of donors relative to control ($p<0.05$). For C), IL-6 mRNA levels with Nutlin-3 plus PFT-α treatment are significantly lower than Nutlin-3 treatment alone ($p<0.05$).

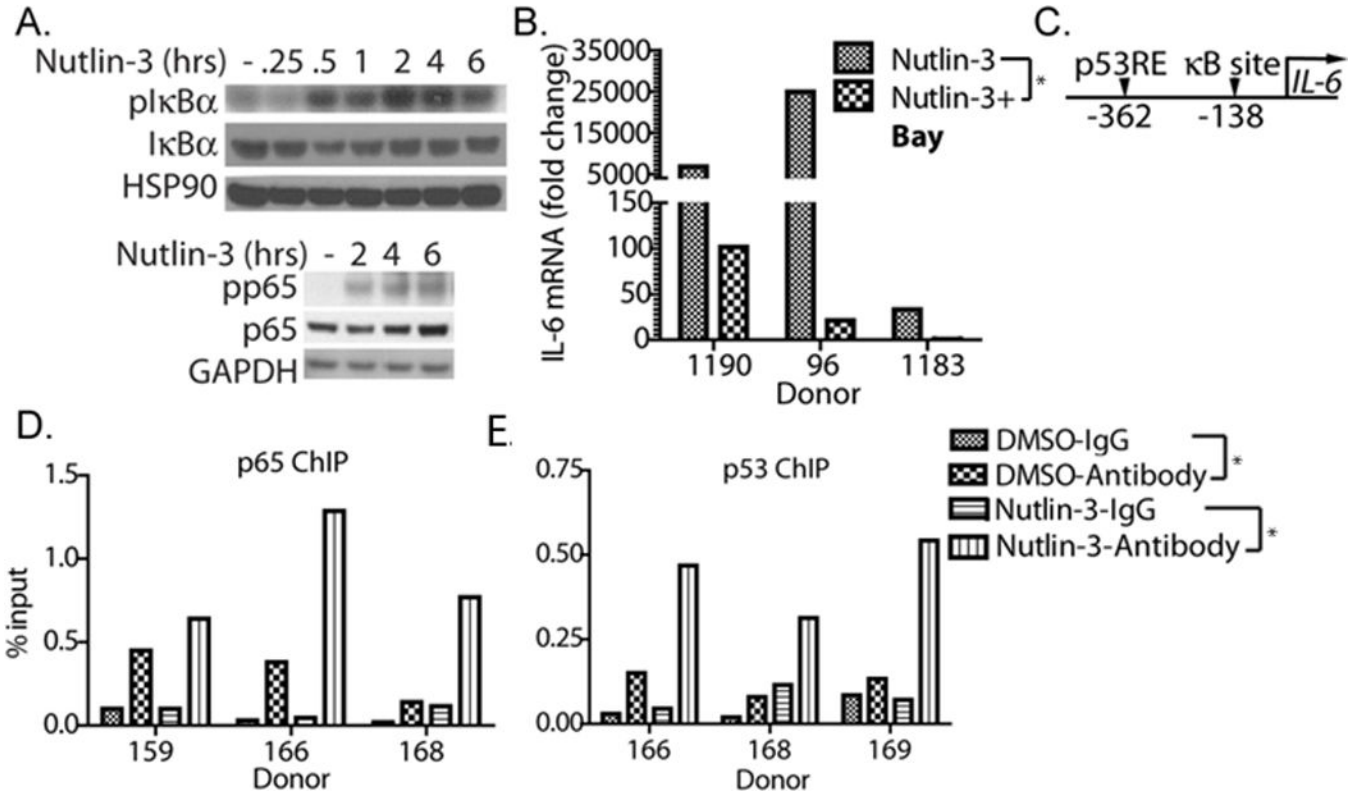

Figure 3. Nutlin-3 triggers activation the NF-κB subunit p65 and binding of p53 and p65 to the promoter of IL-6

A) Immunoblot analysis was performed to measure protein levels from Nutlin-3 treated and DMSO treated ("-") macrophages. Upper panel: phosphorylated IκBα ("p IκBα" Ser32 and Ser36) and total IκBα protein were measured in cytoplasmic extracts from monocyte-derived macrophages. Lower panel: phosphorylated p65 ("pp65," Ser276) and total p65 protein were measured from whole cell extracts from monocyte-derived macrophages. B) Fold-change in IL-6 mRNA levels from macrophages treated with Nutlin-3 for 2 hours with and without pretreatment of Bay-11-7083 ("Bay," 30 minutes). IL-6 mRNA levels with Nutlin-3 plus Bay-11-7082 for the group of donors are significantly lower than Nutlin-3 treatment alone ($p<0.05$). C) Schematic of the location of the p53 half site and κB site in the IL-6 promoter region relative to the TSS. D) p65 ChIP or E) p53 ChIP of monocyte-derived macrophages were treated with Nutlin-3 or DMSO for 1 hour. Primers that amplified the IL-6 promoter region were used in a PCR reaction with DNA collected from each ChIP. Values are plotted as % Input. Enriched binding of p65 and p53 to the IL-6 promoter is significantly greater (*$p<0.05$) than with IgG in all samples (i.e., with DMSO and Nutlin-3 treatment).

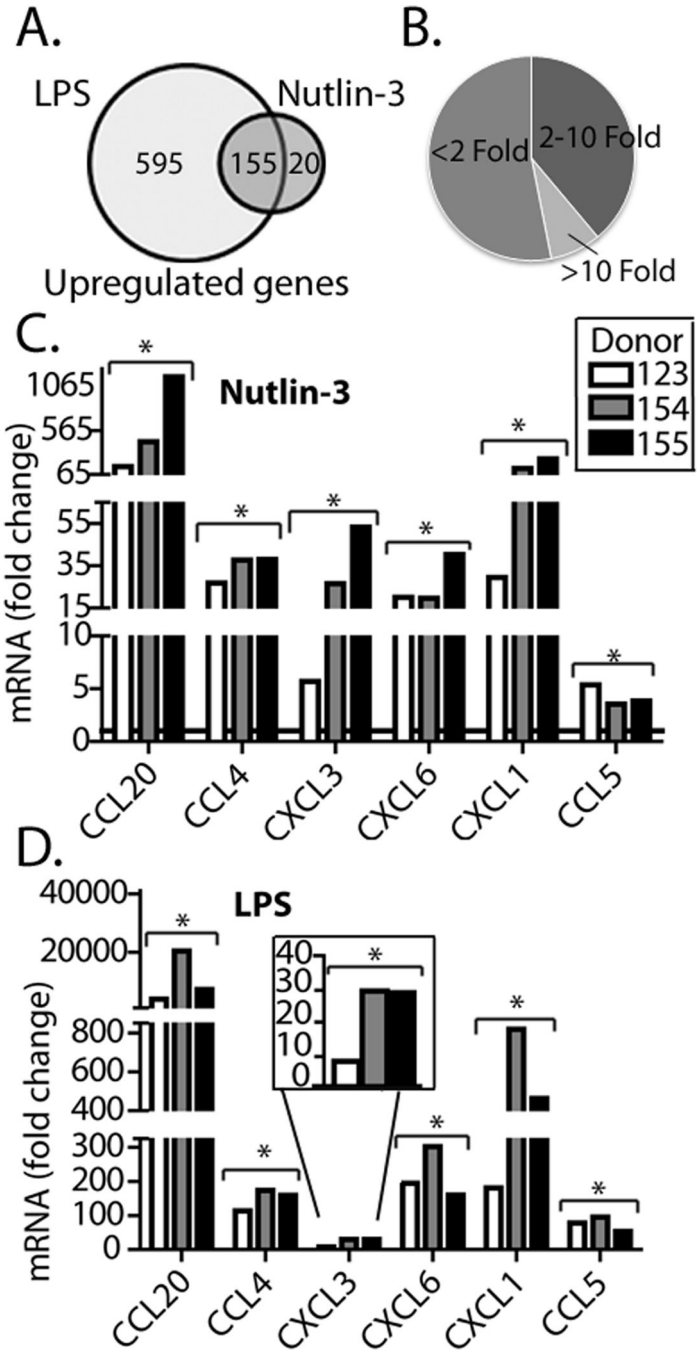

Figure 4. Microarray analysis of Nutlin-3 and LPS treated monocyte-derived macrophages

A) A Venn diagram shows the overlap of Nutlin-3 and LPS upregulated genes. B) A pie chart shows relative quantities of genes in each group (within the 155 gene subset) based on the level of fold change. (C-D) Validation of selected highly induced pro-inflammatory chemokine genes after C) Nutlin-3 and D) LPS treatment in monocyte-derived macrophages from three individual donors with RT-PCR. Values for CXCL3 induction by LPS are depicted in the inset. Values for each donor are depicted, and, when grouped, the mRNA level of each gene is significantly higher with C) Nutlin-3 and D) LPS treatments compared to C) DMSO-treated and D) untreated controls (*$p<0.05$).

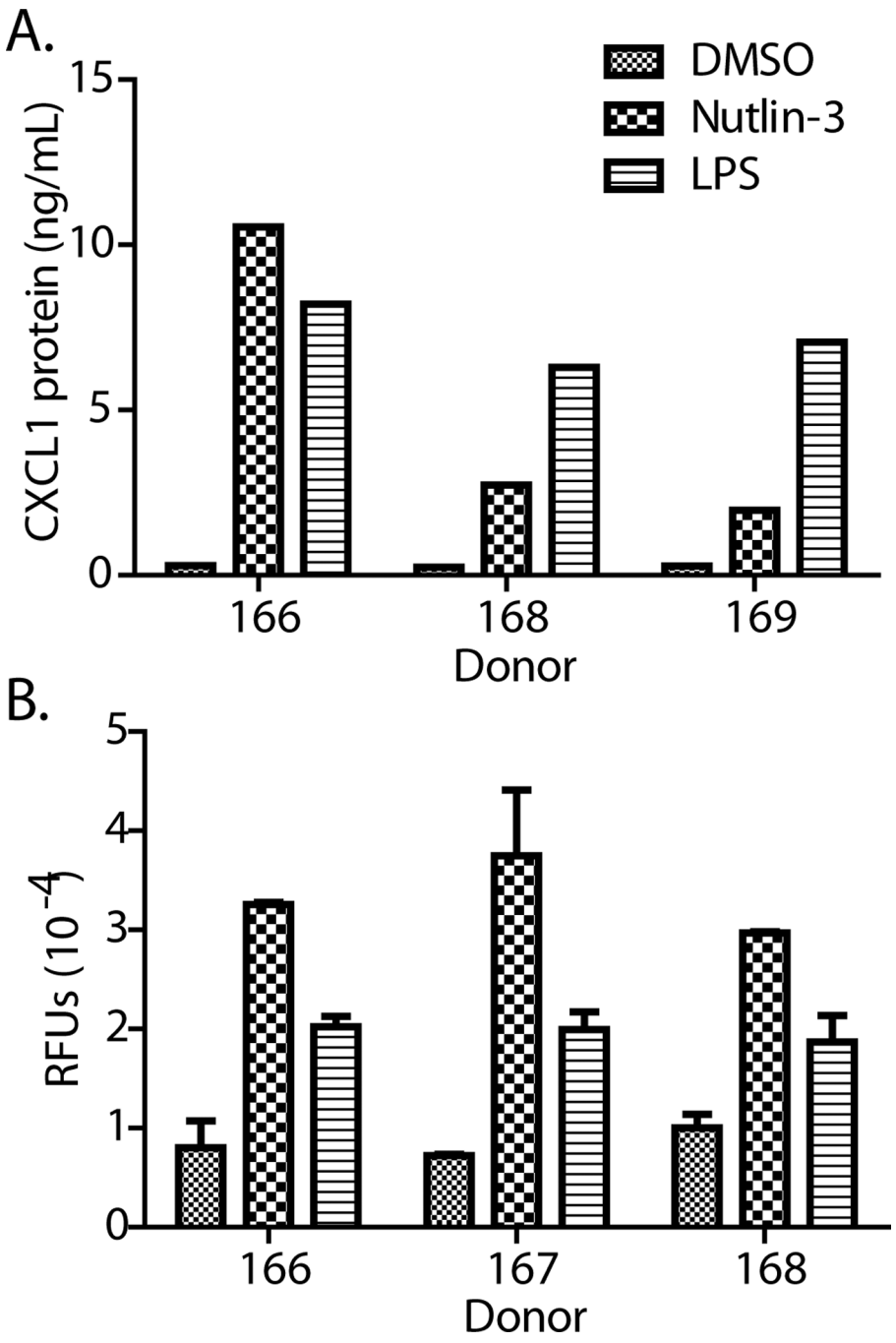

Figure 5. Nutlin-3 induces CXCL1 secretion and promotes human neutrophil migration

A) CXCL1 secreted protein from monocyte-derived macrophages treated with DMSO, Nutlin-3, or LPS for 24 hours was measured with an ELISA. Values for each donor are depicted, and, when grouped the CXCL1 protein levels are significantly higher in Nutlin-3 and LPS treated samples compared to untreated controls ($p<0.05$). For the duration of the Nutlin-3 and LPS treatments, the culture medium was not supplemented with serum. B) Migration of neutrophils towards supernatants from A. Neutrophil migration toward supernatants is plotted for each donor from Nutlin-3- and LPS-treated macrophages normalized to untreated controls. RFU = Relative Fluorescence Units. When grouped, migration toward supernatants from Nutlin-3 and LPS treated cells is significantly higher than towards that from untreated cells ($P<0.05$).

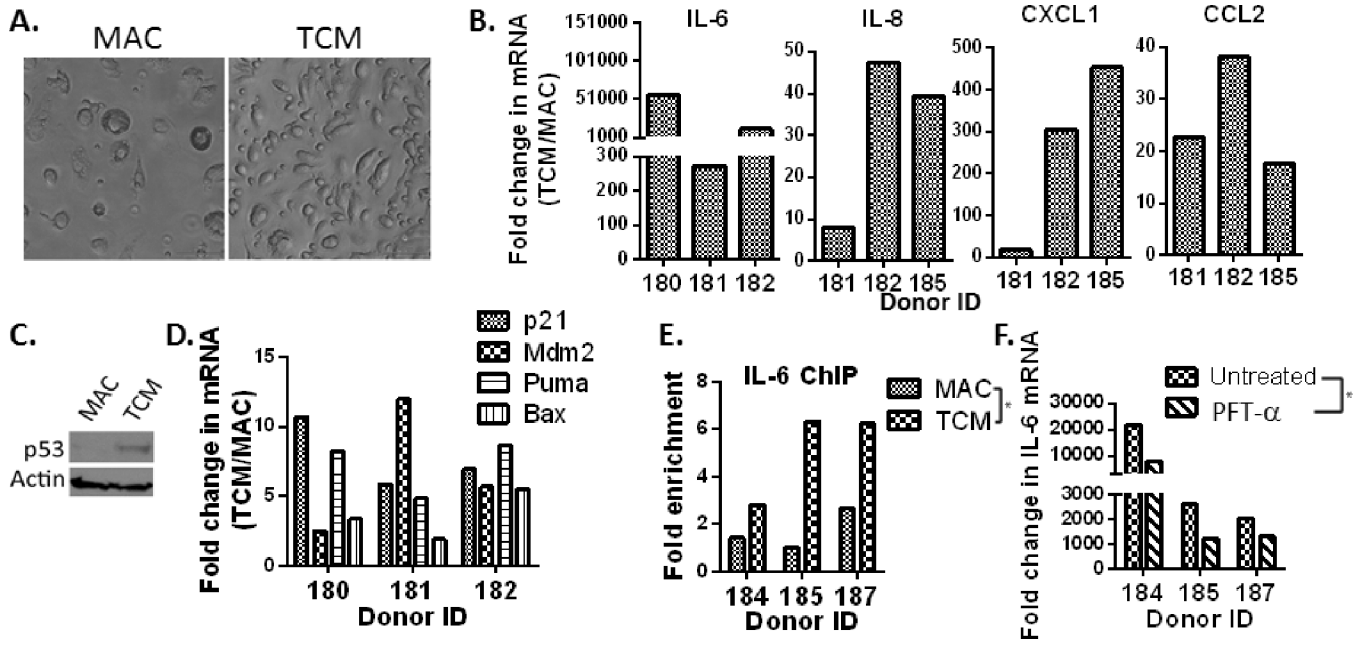

Figure 6. p53 drives high basal levels of IL-6 in TAM-like TCMs

A) Micrograph of normal macrophages ("MAC") and TCMs shows different morphologies. B) RT-PCR analysis of TAM-like expression markers indicated above each graph in TCMs normalized to MACs. Values from each donor are plotted, and, when grouped, the mRNA level for each gene shown is significantly higher ($p<0.05$) in TCMs compared to MACs. C) Immunoblot analysis of levels of p53 in MACs and TCMs. D) RT-PCR analysis of the indicated p53 target genes in TCMs normalized to MACs. When grouped by donors, $p<0.05$ for each gene shown. E) p53 binding to the IL-6 promoter region by ChIP analysis in TCMs and MACs (plotted as Fold enrichment of p53 ChIP over IgG ChIP signals). For the group, the TCM values are significantly higher than MAC values, $p<0.05$. F) RT-PCR analysis of IL-6 mRNA levels TCMs either untreated or treated with PFT-α for 4 hours. Data are graphed as fold change (PFT-α/untreated). PFT-α treatment significantly reduced IL-6 levels, $p<0.05$. In all data sets, TCMs are compared to MACs from the same donor.

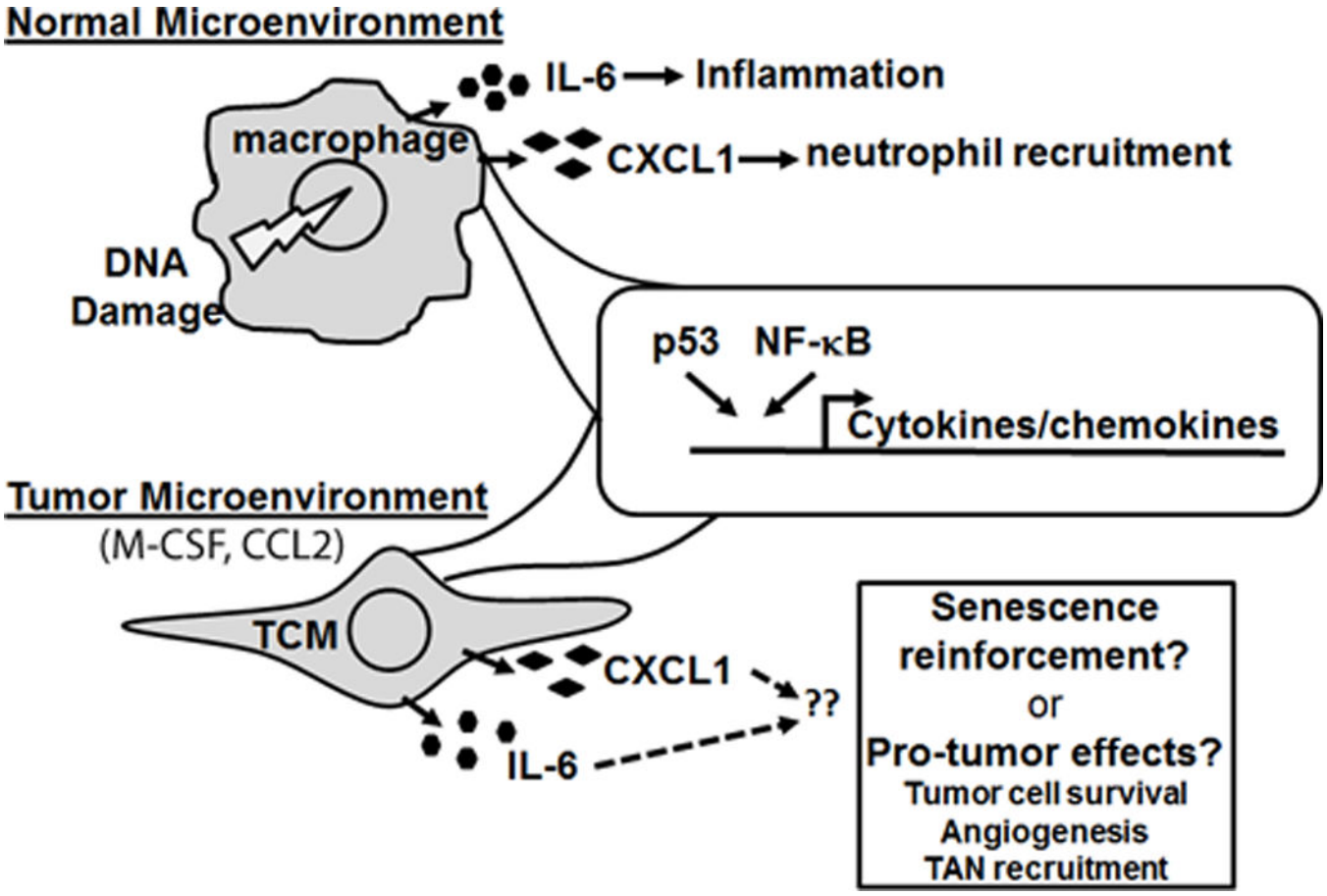

Figure 7. Schematic of p53 and NF-κB signaling and interaction in human macrophages

p53 and NF-κB are activated in macrophages after challenge with stressors (like DNA damaging agents and Nutlin-3) in macrophages in the normal microenvironment and in macrophages associated with the tumor microenvironment. In both types of macrophages, p53 and NF-κB activation results in induction of pro-inflammatory cytokines and chemokines by binding of both factors to promoter regions of these genes. Biological outcomes as a result of p53/NF-κB co-regulation of these genes like IL-6 and IL-8 are likely distinct depending on the microenvironment. In a normal microenvironment, IL-6 and CXCL1 results in inflammation and neutrophil recruitment, whereas these proteins may have pro-tumor roles in a tumor microenvironment such as enhancing tumor cell survival and Tumor Associated Neutrophil (TAN) recruitment.